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3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	**NORCHIP AS** **INDUSTRIVEIEN 8** **N-3490 KLOKKARSTUA** **NORWAY** 7946734001
	Patents ADP number *(if you know it)*	
	If the applicant is a corporate body, give the country/state of its incorporation	**NORWAY**
4.	Title of the invention	**METHOD FOR DETECTING HUMAN PAPILLOMAVIRUS mRNA**
5.	Name of your agent *(if you have one)*	**BOULT WADE TENNANT**
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	**VERULAM GARDENS** **70 GRAY'S INN ROAD** **LONDON WC1X 8BT**
	Patents ADP number *(if you know it)*	**42001**
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12. Name and daytime telephone number of person to contact in the United Kingdom

Dr Nina L White
020 7430 7500

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DUPLICATE

METHOD FOR DETECTING HUMAN PAPILLOMAVIRUS mRNA

Field of the invention

The present invention relates to *in vitro* methods of screening human subjects in order to assess their risk of developing cervical carcinoma.

Background to the invention

Cervical carcinoma is one of the most common malignant diseases world-wide and is one of the leading causes of morbidity and mortality among women (Parkin DM, Pisani P, Ferlay J (1993) Int J Cancer 54: 594-606;Pisani P, Parkin DM, Ferlay J (1993) Int J Cancer 55: 891-903). 15,700 new cases of invasive cervical cancer were predicted in the United States in 1996, and the annual world-wide incidence is estimated to be 450,000 by the World Health Organization (1990). The annual incidence rate differs in different parts of the world, ranging from 7.6 per 100,000 in western Asia to 46.8 per 100,000 in southern Africa (Parkin et al., 1993 *ibid*).

The current conception of cervical carcinoma is that it is a multistage disease, often developing over a period of 10-25 years. Invasive squamous-cell carcinoma of the cervix is represented by penetration through the basal lamina and invading the stroma or epithelial lamina propria. The clinical course of cervical carcinoma shows considerable variation. Prognosis has been related to clinical stage, lymph node involvement, primary tumour mass, histology type, depth of invasion and lymphatic permeation (Delgado G, et al., (1990) Gynecol Oncol 38: 352-357). Some patients with less favourable tumour characteristics have a relatively good outcome, while others suffer a fatal outcome of an initially limited disease. This

shows a clear need for additional markers to further characterise newly diagnosed cervical carcinomas, in order to administer risk-adapted therapy (Ikenberg H, et al., Int. J. Cancer 59:322-6. 1994).

The epidemiology of cervical cancer has shown strong association with religious, marital and sexual patterns. Almost 100 case-control studies have examined the relationship between HPV and cervical neoplasia and almost all have found positive associations (IARC monographs, 1995). The association is strong, consistent and specific to a limited number of viral types (Munoz N, Bosch FX (1992) HPV and cervical neoplasia: review of case-control and cohort studies. IARC Sci Publ 251-261). Among the most informative studies, strong associations with HPV 16 DNA have been observed with remarkable consistency for invasive cancer and high-grade CIN lesions, ruling out the possibility that this association can be explained by chance, bias or confounding (IARC monographs, 1995). Indirect evidence suggested that HPV DNA detected in cancer cells is a good marker for the role of HPV infection earlier in the carcinogenesis. Dose-response relationship has been reported between increasing viral load and risk of cervical carcinoma (Munoz and Bosch, 1992 *ibid*). In some larger series up to 100% of the tumours were positive for HPV but the existence of virus-negative cervical carcinomas is still debatable (Meijer CJ, et al., (1992) Detection of human papillomavirus in cervical scrapes by the polymerase chain reaction in relation to cytology: possible implications for cervical cancer screening. IARC Sci Publ 271-281; Das BC, et al., (1993) Cancer 72: 147-153).

The most frequent HPV types found in squamous-cell cervical carcinomas are HPV 16 (41%-86%)

and 18 (2%-22%). In addition HPV 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 61, 66 and 68 are also found (IARC, monographs, 1995). In the HPV2000 International conference in Barcelona HPV 16, 18, 31 and 45 were defined as high risk, while HPV 33, 35, 39, 51, 52, 56, 58, 59, 68 were defined as intermediate risk (Keerti V. Shah. P71). The 13 high risk plus intermediate risk HPVs are together often referred to as cancer-associated HPV types.

A number of studies have explored the potential role of HPV testing in cervical screening (see Cuzick et al. A systematic review of the role of human papillomavirus testing withing a cervical screening programme. Health Technol Assess 3:14. 1999).

Reid et al., (Reid R, et al., (1991) Am J Obstet Gynecol 164: 1461-1469) where the first to demonstrate a role for HPV testing in a screening context. This study was carried out on high-risk women from sexually transmitted disease clinics and specialist gynaecologists, and used a sensitive (low stringency) Southern blot hybridisation for HPV detection. A total of 1012 women were enrolled, and cervicography was also considered as a possible adjunct to cytology. Twenty-three CIN II/III lesions were found altogether, but only 12 were detected by cytology (sensitivity 52%, specificity 92%). HPV testing found 16 high-grade lesions.

Bauer et al. (Bauer HM, et al., (1991) JAMA 265: 472-477) report an early PCR-based study using MY09/11 primers (Manos M, et al.,(1990) Lancet 335: 734) in young women attending for routine smears (college students). They found a positive rate of 46% in 467 women, which was much higher than for dot blot assay (11%).

In a study using PCR with GP5/6 primers (Van Den Brule AJ, et al., (1990) J Clin Microbiol 28: 2739-2743) van der Brule et al. (Van Den Brule AJ, et al., (1991) Int J Cancer 48: 404-408) showed a very strong correlation of HPV positivity with cervical neoplasia as assessed by cytology. In older women (aged 35-55 years) with negative cytology the HPV positivity rate was only 3.5%, and this was reduced to 1.5% if only types 16, 18, 31 and 33 were considered, while women with histological carcinoma in situ were all HPV-positive, and 90% had one of the four above types. Women with less severe cytological abnormalities had lower HPV positivity rates in a graded way, showing a clear trend.

Roda Housman et al. (Roda Housman AM, et al., (1994) Int J Cancer 56: 802-806) expanded these observations by looking at a further 1373 women with abnormal smears. This study also confirmed increasing positivity rate with increasing severity of smear results. They also noted that the level of HPV heterogeneity decreased from 22 types for low-grade smears to ten "high-risk" types for high grade smears. This paper did not include any cytologically negative women, nor was cytological disease confirmed histologically.

Cuzick et al. (Cuzick J, et al., (1992) Lancet 340: 112-113; Cuzick J, et al., (1994) Br J Cancer 69: 167-171) were the first to report that HPV testing provided useful information for the triage of cytological abnormalities detected during random screening. In a study of 133 women, referral for coloposcopy they found a positive predictive value of 42%, which was similar to that for moderate dyskaryosis. The results were most striking for HPV 16, where 39 of 42 HPV 16 positive women were found to

have high-grade CIN on biopsy. This study pointed out the importance of assessing viral load and only considered high levels of high-risk types as positive.

Cox et al. (Cox JT, et al., (1995) Am J Obstet Gynecol 172: 946-954) demonstrated a role for HPV testing using the Hybrid Capture™ system (DIGENE Corporation, Gaithersburg, MD, USA) for triaging women with borderline smears. This test was performed on 217 such women from a college referral service, and a sensitivity of 93% was found for CINII/III compared with 73% for repeat cytology. High viral load was found to further improve performance by reducing false positives. When 5 RLU was taken as a cut-off, a PPV of approximately 24% was found with no loss of sensitivity.

Cuzick et al. (Cuzick J, et al., (1995) Lancet 345: 1533-1536) evaluated HPV testing in a primary screening context in 1985 women attending for routine screening at a family planning clinic. Sensitivity using type-specific PCR for the four common HPV types (75%) exceeded that of cytology (46%), and the PPV for a positive HPV test (42%) was similar to that for moderate dyskaryosis (43%).

WO 91/08312 describes methods for determining the prognosis of individuals infected with HPV which comprise measuring the level of HPV activity by detecting transcripts of all or a portion of the E6 and/or E7 HPV genes in a sample and comparing the measurements of HPV activity with a previously established relationship between activity and risk of progression to serious cervical dysplasia or carcinoma.

WO 99/29890 describes methods for the assessment

of HPV infection based on the measurement and analysis of gene expression levels. In particular, WO 99/29890 describes methods which are based on measuring the levels of expression of two or more HPV genes (e.g. HPV E6, E7, L1 and E2) and then comparing the ratio of expression of combinations of these genes to provide an indication of the stage of HPV-based disease in a patient.

The present inventors have determined that it is possible to make a clinically useful assessment of HPV-associated disease based only on a simple positive/negative determination of expression of HPV L1 and E6 mRNA transcripts, with no requirement for accurate quantitative measurements of expression levels or for determination of differences in the levels of expression of the two transcripts. This method is technically simple and, in a preferred embodiment, is amenable to automation in a mid-to-high throughput format. Furthermore, on the basis of results obtained using the method of the invention the inventors have defined a novel scheme for classification of patients on the basis of risk of developing cervical carcinoma which is related to disease-relevant molecular changes in the pattern of HPV gene expression and is independent of CIN classification.

Therefore, in a first aspect the invention provides an *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or full length E6 mRNA are scored as being at risk of developing cervical carcinoma.

A positive screening result in the method of the invention is indicated by positive expression of L1 mRNA and/or E6 mRNA. Positive expression of either one of these mRNAs or both mRNAs is taken as an indication that the subject is "at risk" for development of cervical carcinoma. Women who express E6 mRNA are at high risk of developing cell changes because oncogenic E6 and E7 bind to cell cycle regulatory proteins and act as a switch for cell proliferation. Clear expression of E6 mRNA provides a direct indication of cell changes in the cervix. Expression of L1 mRNA, with or without expression of E6 mRNA is also indicative of the presence of an active HPV.

In the wider context of cervical screening, women identified as positive for L1 and/or E6 mRNA expression may be selected for further investigation, for example using cytology. Thus, at one level the method of the invention may provide a technical simple means of pre-screening a population of women in order to identify HPV-positive subjects who may be selected for further investigation.

In the method of the invention "positive expression" of an mRNA is taken to mean expression above background. There is no absolute requirement for accurate quantitative determination of the level of mRNA expression or for accurate determination of the relative levels of expression of L1 and E6 mRNA.

In one embodiment, the method may comprise a quantitative determination of levels of mRNA expression. In a preferred embodiment in order to provide a clear distinction between "positive expression" and "negative expression" a determination of "positive expression" may require the presence of

more than 50 copies of the relevant mRNA (per ml of sample or per total volume of sample), whereas a determination of "negative expression" may require the presence of less than 1 copy of the relevant mRNA (per ml of sample or per total volume of sample).

In a further preferred embodiment, the method may involve screening for E6 mRNA using a technique which is able to detect specifically E6 mRNA from cancer-associated HPV types, more preferably "high risk" cancer-associated HPV types. In the most preferred embodiment the method involves screening for E6 mRNA using a technique which is able to detect E6 mRNA from HPV types 16, 18, 31 and 33. Most preferably, the method will specifically detect expression of E6 mRNA from at least one of HPV types 16, 18, 31 and 33, and preferably all four types. However, women positive for positive for expression of E6 from other types than 16, 18, 31 and 33, e.g. 35, 39, 45, 52, 56, 58, 59, 66 and 68 may still be 'at risk' of developing cervical carcinoma. Thus, the method may encompass screening for expression of E6 mRNA from one or more of these HPV types, most preferably in addition to screening for E6 mRNA from HPV types 16, 18, 31 and 33. Certain HPV types exhibit a marked geographical/population distribution. Therefore, it may be appropriate to include primers specific for an HPV type known to be prevalent in the population/geographical area under test, for example in addition to screening for HPV types 16, 18, 31 and 33.

For the avoidance of doubt, the term "E6 mRNA" encompasses all naturally occurring mRNA transcripts which contain all or part of the E6 open reading frame, including naturally occurring splice variants, and therefore includes transcripts which additionally

contain all or part of the E7 open reading frame (and indeed further open reading frames). Four E6/E7 mRNA species have so far been described in cells infected with HPV 16, namely an unspliced E6 transcript and three spliced transcripts denoted E6*I, E6*II and E6*III (Smotkin D, et al., J Virol. 1989 Mar 63(3):1441-7; Smotkin D, Wettstein FO. Proc Natl Acad Sci USA. 1986 Jul 83(13):4680-4; Doorbar J. et al., Virology. 1990 Sep 178(1):254-62; Cornelissen MT, et al. J Gen Virol. 1990 May 71(Pt 5):1243-6; Johnson MA, et al. J Gen Virol. 1990 Jul 71(Pt 7):1473-9; Schneider-Maunoury S, et al. J Virol. 1987 Oct 61(10):3295-8; Sherman L, et al. Int J Cancer. 1992 Feb 50(3):356-64). All four transcripts are transcribed from a single promoter (p97) located just upstream of the second ATG of the E6 ORF.

In one embodiment the method may comprise screening for E6 transcripts which contain all or part of the E7 open reading frame, This may be accomplished, for example, using primers or probes specific for the E7 coding region.

In a further embodiment, the method may comprise screening for the presence of "full length" E6 transcripts. In the case of HPV 16 the term "full length E6 transcripts" refers to transcripts which contain all of the region from nucleotide (nt) 97 to nt 880 in the E6 ORF, inclusive of nt 97 <u>and</u> 880. Nucleotide positions are numbered according to standard HPV nomenclature (see Human Papillomavirus Compendium OnLine, available via the internet or in paper form from HV Database, Mail Stop K710, Los Alamos National Laboratory, Los Alamos, NM 87545, USA). Specific detection of full length transcripts may be accomplished, for example, using primers or probes which are specific for the region which is

present only in full length E6 transcripts, not in splice variants. Different HPV types exhibit different patterns of E6/E7 mRNA expression. Transcript maps for various HPV types, including HPV types 16 and 31, which may be used to assist in the design of probes or primers for detection of E6/E7 transcripts are publicly available via the Human Papillomavirus Compendium (as above).

E6 oligonucleotide primers are described herein which are suitable for use in amplification of regions of the E6 mRNA from various HPV types by NASBA or PCR.

In a preferred embodiment the method of the invention may involve screening for L1 mRNA expression using a technique which is able to detect L1 mRNA from substantially all known HPV types or at least the major cancer-associated HPV types (e.g. preferably all of HPV types 16, 18, 31 and 33) . L1 primers and probes are described herein which are capable of detecting L1 mRNA from HPV types 6, 11, 16, 18, 31, 33, 35 and 51 in cervical samples.

Detection of L1 transcripts can be said to detect HPV "virulence", meaning the presence of HPV lytic activity. Detection of E6/E7 transcripts can be said to detect HPV "pathogenesis" since expression of these mRNAs is indicative of molecular events associated with risk of developing carcinoma.

In a study of 4589 women it was possible to detect all except one case of CIN III lesions or cancer using the method of the invention (see accompanying Examples).

In a specific embodiment, the method of the invention may be used to classify subjects into four

different classes of risk for developing cervical carcinoma on the basis of positive/negative scoring of expression of L1 and E6 mRNA.

Accordingly, in a further aspect the invention provides an *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts of the L1 gene of HPV and mRNA transcripts of the E6 gene of HPV, and sorting the subject into one of four categories of risk for development of cervical carcinoma based on expression of L1 and/or E6 mRNA according to the following classification:

Risk category 1: subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 or 68. Those individuals positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31 or 33 are scored as being at higher risk, for example in comparison to individuals negative for these types but positive for expression of E6 mRNA from at least one of HPV types 35, 39, 45, 52, 56, 58, 59, 66 or 68.

Risk category 2: subjects positive for expression of L1 mRNA and positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 or 68. Those individuals positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31 or 33 are scored as being at higher risk, for example in comparison to individuals negative for these types but positive for expression of E6 mRNA from at least one of HPV types 35, 39, 45, 52, 56, 58, 59, 66 or 68.

Risk category 3: subjects positive for expression of L1 mRNA but negative for expression of E6 mRNA from the cancer-associated HPV types, (e.g. negative for expression of E6 mRNA from HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68).

Risk category 4: subjects negative for expression of L1 mRNA and negative for expression of E6 mRNA.

In a preferred embodiment, positive expression is indicated by the presence of more than 50 copies of the transcript per ml (or total volume of the sample) and negative expression is indicated by the presence of less than 1 copy of the transcript per ml (or total volume of the sample).

The above classification is based on molecular events which are relevant to risk of developing cervical carcinoma and is independent of the CIN status of the subjects. Thus, this method of classification may provide an alternative to the use of cytology in the routine screening of women to identify those at potential risk of developing cervical carcinoma. The method may also be used as an adjunct to cytology, for example as a confirmatory test to confirm a risk assessment made on the basis of cytology.

Women positive for expression of high risk E6 mRNA from one of HPV types 16, 18, 31 or 33 but negative for expression of L1 are in the highest level of risk of developing severe cell changes and cell abnormalities. This is due to the fact that a negative result for L1 mRNA expression is directly indicative of integrated HPV, and therefore a higher probability of high and constant expression of E6 and E7. Integration of a virus in the human genome has

also a direct impact on the stability of the cells. Integration of HPV also reduces the possibility of regression of cell changes.

Women positive for expression of E6 mRNA from one of HPV types 16, 18, 31 or 33 and positive for expression of L1 mRNA have a "high risk" HPV expression and it is still possible that the HPV has been integrated. However, the risk of these women is not classed as high as those who are L1 negative and E6 positive, since there is a reasonable probability that they do not have integrated HPV.

Women negative for expression of E6 mRNA from HPV types 16, 18, 31 or 33 but positive for expression of E6 mRNA from another HPV type, e.g. 35, 39, 45, 52, 56, 58, 59, 66 and 68, are still considered "at risk" and may therefore be placed in risk categories 1 or 2 (as defined above) depending on whether they are positive or negative for expression of L1 mRNA.

Women positive for L1 mRNA but negative for E6 mRNA are scored as being at moderate risk. There may be high-risk HPV types in the sample and L1 expression is indicative of lytic activity. There may also be integrated HPV types but only with viruses that are rare. However, detection of lytic activity may show that the cell may soon develop some changes.

In the wider context of cervical screening the method of the invention may be used to classify women according to risk of developing cervical carcinoma and therefore provide a basis for decisions concerning treatment and/or further screening. By way of example: women in risk category 1, particularly those who exhibit positive expression of E6 mRNA from at least one of HPV types 16, 18, 31 or 33, might be

identified as requiring "immediate action", meaning conisation or colposcopy, including a biopsy and histology.

Women in risk category 2, as defined above, might be scored as requiring immediate attention, meaning colposcopy alone or colposcopy including a biopsy and histology.

Women in risk category 3, as defined above, might be scored as requiring immediate re-test, meaning recall for a further test for HPV expression immediately or after a relatively short interval, e.g. six months.

Women in risk category 4, as defined above, might be returned to the screening program, to be re-tested for HPV expression at a later date.

In a further specific embodiment the invention provides an *in vitro* method of screening human subjects for the presence of integrated HPV, which method comprises screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA are scored as carrying integrated HPV.

As aforesaid, the presence of integrated HPV is indicated by a negative result for L1 mRNA expression, together with a positive result for expression of E6 mRNA. Therefore, the ability to predict the presence of integrated HPV is critically dependent on the ability to score a negative result for L1 mRNA expression. This requires a detection technique which has maximal sensitivity, yet produces minimal false-

negative results. In a preferred embodiment this is achieved by using a sensitive amplification and real-time detection technique to screen for the presence or absence of L1 mRNA. The most preferred technique is real-time NASBA amplification using molecular beacons probes, as described by Leone et al., Nucleic Acids Research., 1998, Vol 26, 2150-2155. Due to the sensitivity of this technique the occurrence of false-negative results is minimised and a result of "negative L1 expression" can be scored with greater confidence.

In further embodiments, the above-described methods of the invention may comprise screening for expression of mRNA transcripts from the human $p16^{ink4a}$ gene, in addition to screening for expression of HPV L1 and E6 transcripts.

A positive result for expression of $p16^{ink4a}$ mRNA is taken as a further indication of risk of developing cervical carcinoma.

$P16^{ink4a}$, and the related family members, may function to regulate the phosphorylation and the growth suppressive activity of the restinoblastoma gene product (RB). In support of this, it has been found that there is an inverse relationship between the expression of $p16^{ink4a}$ protein and the presence of normal RB in selected cancer cell lines; $p16^{ink4a}$ protein is detectable when RB is mutant, deleted, or inactivated, and it is markedly reduced or absent in cell lines that contain a normal RB. Kheif et al.(Kheif SN et al., Proc. Natl. Acad. Sci. USA 93:4350-4354. 1996), found that $p16^{ink4a}$ protein is expressed in human cervical carcinoma cells that contain either a mutant RB or a wild-type RB that is functionally inactivated by E7. They also show that

the inactivation of RB correlates with an upregulation of p16^{ink4a} confirming a feedback loop involving p16^{ink4a} and RB. Milde-Langosch et al. (Milde-Langosch K, et al., (2001) Virchows Arch 439: 55-61) found that there were significant correlations between strong p16 expression and HPV16/18 infection and between strong p16 expression and HPV 16/18 E6/E7 oncogene expression. Klaes et al., (Klaes R, et al., (2001) Int J Cancer 92: 276-284) observed a strong over expression of the p16^{ink4a} gene product in 150 of 152 high-grade dysplastic cervical lesions (CIN II to invasive cancer), whereas normal cervical epithelium or inflammatory or metaplastic lesions were not stained with the p16^{ink4a} specific monoclonal antibody E6H4. All CIN I scored lesions associated with LR-HPV types displayed no or only focal or sporadic reactivity, whereas all but two CIN I scored lesions associated with HR-HPV types showed strong and diffuse staining for p16^{ink4a}.

The disclosed screening methods may be carried out on a preparation of nucleic acid isolated from a clinical sample or biopsy taken from the subject under test. Suitable samples which may be used as a source of nucleic acid include (but not exclusively) cervical swabs, cervical biopsies, cervical scrapings, skin biopsies/warts, also paraffin embedded tissues, formalin or methanol fixed cells.

The preparation of nucleic acid to be screened using the disclosed method must include mRNA, however it need not be a preparation of purified poly A+ mRNA and preparations of total RNA or crude preparations of total nucleic acid containing both RNA and genomic DNA, or even crude cell lysates are also suitable as starting material for a NASBA reaction. Essentially any technique known in the art for the isolation of a

preparation of nucleic acid including mRNA may be used to isolate nucleic acid from a test sample. A preferred technique is the "Boom" isolation method described in US-A-5,234,809 and EP-B-0389,063. This method, which can be used to isolate a nucleic acid preparation containing both RNA and DNA, is based on the nucleic acid binding properties of silicon dioxide particles in the presence of the chaotropic agent guanidine thiocyanate (GuSCN).

The methods of the invention are based on assessment of active transcription of the HPV genome. The methods are not limited with respect to the precise technique used to detect mRNA expression. Many techniques for detection of specific mRNA sequences are known in the art and may be used in accordance with the invention. For example, specific mRNAs may be detected by hybridisation, amplification or sequencing techniques.

It is most preferred to detect mRNA expression by means of an amplification technique, most preferably an isothermal amplification such as NASBA, transcription-mediated amplification, signal-mediated amplification of RNA technology, isothermal solution phase amplification, etc. All of these methods are well known in the art More preferably mRNA expression is detected by an isothermal amplification in combination with real-time detection of the amplification product. The most preferred combination is amplification by NASBA, coupled with real-time detection of the amplification product using molecular beacons technology, as described by Leone et al., Nucleic Acids Research, 1998, Vol 26, 2150-2155.

Methods for the detection of HPV in a test sample using the NASBA technique will generally comprise the

following steps:

(a) assembling a reaction medium comprising suitable primer-pairs, an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter, and ribonucleoside and deoxyribonucleoside triphosphates;

(b) incubating the reaction medium with a preparation of nucleic acid isolated from a test sample suspected of containing HPV under reaction conditions which permit a NASBA amplification reaction; and

(c) detecting and/or quantitatively measuring any HPV-specific product of the NASBA amplification reaction.

Detection of the specific product(s) of the NASBA reaction (i.e. sense and/or antisense copies of the target RNA) may be carried out in a number of different ways. In one approach the NASBA product(s) may be detected with the use of an HPV-specific hybridisation probe capable of specifically annealing to the NASBA product. The hybridisation probe may be attached to a revealing label, for example a fluorescent, luminescent, radioactive or chemiluminescent compound or an enzyme label or any other type of label known to those of ordinary skill in the art. The precise nature of the label is not critical, but it should be capable of producing a signal detectable by external means, either by itself or in conjunction with one or more additional substances (e.g. the substrate for an enzyme).

A preferred detection method is so-called "real-time NASBA" which allows continuous monitoring of the formation of the product of the NASBA reaction over

the course of the reaction. In a preferred embodiment this may be achieved using a "molecular beacons" probe comprising an HPV-specific sequence capable of annealing to the NASBA product, a stem-duplex forming oligonucleotide sequence and a pair of fluorescer/quencher moieties, as known in the art and described herein. If the molecular beacons probe is added to the reaction mixture prior to amplification it may be possible to monitor the formation of the NASBA product in real-time (Leone *et al.*, Nucleic Acids Research, 1998, Vol 26, 2150-2155). Reagent kits and instrumentation for performing real-time NASBA detection are available commercially (e.g. NucliSens™ EasyQ system, from Organon Teknika).

In a further approach, the molecular beacons technology may be incorporated into the primer 2 oligonucleotide allowing real-time monitoring of the NASBA reaction without the need for a separate hybridisation probe.

In a still further approach the products of the NASBA reaction may be monitored using a generic labelled detection probe which hybridises to a nucleotide sequence in the 5' terminus of the primer 2 oligonucleotide. This is equivalent to the "NucliSens™" detection system supplied by Organon Teknika. In this system specificity for NASBA products derived from the target HPV mRNA may be conferred by using HPV-specific capture probes comprising probe oligonucleotides as described herein attached to a solid support such as a magnetic microbead. Most preferably the generic labelled detection probe is the ECL™ detection probe supplied by Organon Teknika. NASBA amplicons are hybridized to the HPV-specific capture probes and the generic ECL probe (via a complementary sequence on primer 2).

Following hybridization the bead/amplicon/ECL probe complexes may be captured at the magnet electrode of an automatic ECL reader (e.g. the NucliSens™ reader supplied by Organon Teknika). Subsequently, a voltage pulse triggers the ECL™ reaction.

The detection of HPV mRNA is also of clinical relevance in cancers other than cervical carcinoma including, for example, head and neck carcinoma, oral and tongue carcinoma, skin carcinoma, anal and vaginal carcinoma. Detection of HPV mRNA may also be very useful in the diagnosis of micrometastases in lymph nodes in the lower part of the body. Hence, the invention also contemplates screens for susceptibility to the above-listed cancers based on screening for expression of HPV L1 and E6 transcripts.

In accordance with a further aspect of the invention there is provided a kit for use in the detection of transcripts of the L1 and E6 genes of HPV, the kit comprising at least one primer-pair suitable for use in amplification of a region of L1 transcripts from at least HPV types 16, 18, 31 and 33 and one or more primer-pairs which enable amplification of a region of E6 transcripts from HPV types 16, 18, 31 and 33.

"Primer-pair" taken to mean are pair of primers which may be used in combination to amplify a specific region of the L1 or E6 mRNA using any known nucleic acid technique. In preferred embodiments the primer-pairs included in the kit will be suitable for use in NASBA amplification or similar isothermal amplification techniques.

The individual primers making up each primer-pair included in the kit may be supplied separately (e.g. a

separate container of each primer) or, more preferably, may be supplied mixed in a single container. Combinations of two or more primer-pairs may be supplied ready-mixed in a single container within the kit. It may be convenient to supply two or more primer-pairs in a single container where the two or more amplification reactions are to be "multiplexed", meaning performed simultaneously in a single reaction vessel.

The primer-pair(s) suitable for use in amplification of a region of E6 transcripts should enable amplification a region of E6 mRNA from at least the major cancer-associated HPV types 16, 18, 31 and 33. There are several different ways in which this can be achieved.

In one embodiment, the kit may contain separate primer-pairs specific for each of HPV types 16, 18, 31 and 33. These primer-pairs may be supplied within the kit in separate containers, or they may be supplied as mixtures of two or more primer-pairs in a single container, for example to enable multiplexing of the amplification reactions.

In a further embodiment, the kit may contain a single primer-pair capable of amplifying a region of the E6 gene from HPV types 16, 18, 31 <u>and</u> 33, which thus enables amplification of all four types in a single amplification reaction. This could, for example, be achieved with the use of a pair of degenerate primers or by selection of a region of the E6 mRNA which is highly conserved across HPV types.

The E6 primer-pair may correspond to any region of the E6 mRNA, an may enable amplification of all or part of the E6 open reading frame and/or the E7 open

reading frame.

The kit may further include primer-pairs suitable for use in amplification of E6 mRNA from HPV types other than types 16, 18, 31 and 33. For example, the kit may be supplemented with E6 primers for detection of an HPV type which is endemic in a particular geographical area or population.

The primer-pair(s) suitable for use in amplification of a region of L1 transcripts should be capable of amplifying a region of L1 mRNA from at least the major cancer-associated HPV types 16, 18, 31 and 33 and will preferably be suitable for use in amplification of a region of L1 mRNAs from substantially all known HPV types. With the use of such primers it is possible to test for active transcription of L1 mRNA from multiple HPV types in a single amplification reaction.

It is possible to design primers capable of detecting L1 transcripts from multiple HPV types by selecting regions of the L1 transcript which are highly conserved.

In a further approach, specificity for multiple HPV types may be achieved with the use of degenerate oligonucleotide primers or complex mixtures of polynucleotides which exhibit minor sequence variations, preferably corresponding to sites of sequence variation between HPV genotypes. The rationale behind the use of such degenerate primers or mixtures is that the mixture may contain at least one primer-pair capable of detecting each HPV type.

In a still further approach specificity for multiple HPV types may be achieved by incorporating

into the primers one or more inosine nucleotides, preferably at sites of sequence variation between HPV genotypes.

The E6 and L1 primer-pairs may be supplied in separate containers within the kit, or the L1 primer-pair(s) may be supplied as a mixture with one or more E6 primer-pairs in a single container.

The kits may further comprise one or more probes suitable for use in detection of the products of amplification reactions carried out using the primer-pairs included within the kit. The probe(s) may be supplied as a separate reagent within the kit. Alternatively, the probe(s) may be supplied as a mixture with one or more primer-pairs.

The primers and probes included in the kit are preferably single stranded DNA molecules. Non-natural synthetic polynucleotides which retain the ability to base-pair with a complementary nucleic acid molecule may also be used, including synthetic oligonucleotides which incorporate modified bases and synthetic oligonucleotides wherein the links between individual nucleosides include bonds other than phosphodiester bonds. The primers and probes may be produced according to techniques well known in the art, such as by chemical synthesis using standard apparatus and protocols for oligonucleotide synthesis.

The primers and probes will typically be isolated single-stranded polynucleotides of no more than 100 bases in length, more typically less than 55 bases in length. For the avoidance of doubt it is hereby stated that the terms "primer" and "probe" exclude naturally occurring full-length HPV genomes.

Several general types of oligonucleotide primers and probes incorporating HPV-specific sequences may be included in the kit. Typically, such primers and probes may comprise additional, non-HPV sequences, for example sequences which are required for an amplification reaction or which facilitate detection of the products of the amplification reaction.

The first type of primers are primer 1 oligonucleotides (also referred to herein as NASBA P1 primers), which are oligonucleotides of generally approximately 50 bases in length, containing an average of about 20 bases at the 3' end that are complementary to a region of the target mRNA. Oligonucleotides suitable for use as NASBA P1 primers are denoted "NASBA P1/PCR" in Table x. The 5' ends of the P1 primer oligonucleotides (represented herein in general terms as X_1) comprise a promoter sequence that is recognized by a specific RNA polymerase. Bacteriophage promoters, for example the T7, T3 and SP6 promoters, are preferred for use in the oligonucleotides of the invention, since they provide advantages of high level transcription which is dependent only on binding of the appropriate RNA polymerase. In a preferred embodiment, the 5' terminal sequence of the P1 primers may comprise the sequence AATTCTAATACGACTCACTATAGGG or the sequence AATTCTAATACGACTCACTATAGGGAGAAGG. These sequences contains a T7 promoter, including the transcription initiation site for T7 RNA polymerase.
The HPV-specific sequences denoted in Table 1 as "NASBA P1/PCR" are suitable for use in both NASBA P1 primers and standard PCR primers. When these sequences are used as the basis of NASBA P1 primers they have the general structure X_1-SEQ, wherein X_1 represents a sequence comprising a promoter and SEQ represents the HPV-specific sequence. The promoter

sequence X_1 is essential. However, when the same sequences are used as the basis of standard PCR primers it is not necessary to include X_1.

A second type of primers are NASBA primer 2 oligonucleotides (also referred to herein as NASBA P2 primers) which generally comprise a sequence of approximately 20 bases substantially identical to a region of the target mRNA. The oligonucleotide sequences denoted in Table x as "NASBA P2/PCR" are suitable for use in both NASBA P1 primers and standard PCR primers.

Oligonucleotides intended for use as NASBA P2 primers may, in a particular but non-limiting embodiment, further comprise a sequence of nucleotides at the 5' end which is unrelated to the target mRNA but which is capable of hybridising to a generic detection probe. The detection probe will preferably be labelled, for example with a fluorescent, luminescent or enzymatic label. In one embodiment the detection probe is labelled with a label that permits detection using ECL™ technology, although it will be appreciated that the invention is in no way limited to this particular method of detection. In a preferred embodiment the 5' end of the primer 2 oligonucleotides may comprise the sequence GATGCAAGGTCGCATATGAG. This sequence is capable of hybridising to a generic ECL™ probe commercially available from Organon Teknika having the following structure:

$Ru(bpy)_3^{2+}$-GAT GCA AGG TCG CAT ATG AG-3'

In a different embodiment the primer 2 oligonucleotide may incorporate "molecular beacons" technology, which is known in the art and described, for example, in WO 95/13399 by Tyagi and Kramer,

Nature Biotechnology. 14: 303-308, 1996, to allow for real-time monitoring of the NASBA reaction.

Target-specific probe oligonucleotides may also be included within the kit. Probe oligonucleotides generally comprise a sequence of approximately 20-25 bases substantially identical to a region of the target mRNA, or the complement thereof. The probe oligonucleotides may be used as target-specific hybridisation probes for detection of the products of a NASBA or PCR reaction. In this connection the probe oligonucleotides may be coupled to a solid support, such as paramagnetic beads, to form a capture probe (see below). In a preferred embodiment the 5' end of the probe oligonucleotide may be labelled with biotin. The addition of a biotin label facilitates attachment of the probe to a solid support via a biotin/streptavidin or biotin/avidin linkage.

Target-specific probes enabling real-time detection of amplification products may incorporate "molecular beacons" technology which is known in the art and described, for example, by Tyagi and Kramer, Nature Biotechnology. 14: 303-308, 1996 and in WO 95/13399.

The term "molecular beacons probes" as used herein is taken to mean molecules having the structure:

X_2-arm_1-target-arm_2-X_3

wherein "target" represents a target-specific sequence of nucleotides, "X_2" and "X_3" represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in

close proximity and "arm_1" and "arm_2" represent complementary sequences capable of forming a stem duplex.

The use of molecular beacons technology allows for real-time monitoring of amplification reactions, for example NASBA amplification (see Leone et al., Nucleic Acids Research., 1998, vol: 26, pp 2150-2155). The molecular beacons probes generally include complementary sequences flanking the HPV-specific sequence, represented herein by the notation arm_1 and arm_2, which are capable of hybridising to each other form a stem duplex structure. The precise sequences of arm_1 and arm_2 are not material to the invention, except for the requirement that these sequences must be capable of forming a stem duplex when the probe is not bound to a target HPV sequence.

Molecular beacons probes also include a fluorescent moiety and a quencher moiety, the fluorescent and the quencher moieties being represented herein by the notation X_2 and X_3. As will be appreciated be the skilled reader, the fluorescer and quencher moieties are selected such that the quencher moiety is capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two moieties are in close proximity, e.g. when the probe is in the hairpin "closed" conformation in the absence of the target sequence. Upon binding to the target sequence, the fluorescent and quencher moieties are held apart such that the fluorescence of the fluorescent moiety is no longer quenched.

Many examples of suitable pairs of quencher/fluorescer moieties which may be used in accordance with the invention are known in the art

(see WO 95/13399, Tyagi and Kramer, ibid). A broad range of fluorophores in many different colours made be used, including for example 5-(2'-aminoethyl)aminonaphthalene-1-sulphonic acid (EDANS), fluorescein, FAM and Texas Red (see Tyagi, Bratu and Kramer, 1998, Nature Biotechnology, 16, 49-53. The use of probes labelled with different coloured fluorophores enables "multiplex" detection of two or more different probes in a single reaction vessel. A preferred quencher is 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), a non-fluorescent chromophore, which serves as a 'universal' quencher for a wide range of fluorophores. The fluorescer and quencher moieties may be covalently attached to the probe in either orientation, either with the fluorescer at or near the 5' end and the quencher at or near the 3' end or vice versa. Protocols for the synthesis of molecular beacon probes are known in the art. A detailed protocol for synthesis is provided in a paper entitled "Molecular Beacons: Hybridization Probes for Detection of Nucleic Acids in Homogenous Solutions" by Sanjay Tyagi et al., Department of Molecular Genetics, Public Health Research Institute, 455 First Avenue, New York, NY 10016, USA, which is available online via the PHRI website (at www.phri.nyu.edu or www.molecular-beacons.org)

Suitable combinations of the NASBA P1 and NASBA P2 primers may be used to drive a NASBA amplification reaction. In order to drive a NASBA amplification reaction the primer 1 and primer 2 oligonucleotides must be capable of priming synthesis of a double-stranded DNA from a target region of mRNA. For this to occur the primer 1 and primer 2 oligonucleotides must comprise target-specific sequences which are complementary to regions of the

sense and the antisense strand of the target mRNA, respectively.

In the first phase of the NASBA amplification cycle, the so-called "non-cyclic" phase, the primer 1 oligonucleotide anneals to a complementary sequence in the target mRNA and its 3' end is extended by the action of an RNA-dependent DNA polymerase (e.g. reverse transcriptase) to form a first-strand cDNA synthesis. The RNA strand of the resulting RNA:DNA hybrid is then digested, e.g. by the action of RNaseH, to leave a single stranded DNA. The primer 2 oligonucleotide anneals to a complementary sequence towards the 3' end of this single stranded DNA and its 3' end is extended (by the action of reverse transcriptase), forming a double stranded DNA. RNA polymerase is then able to transcribe multiple RNA copies from the now transcriptionally active promoter sequence within the double-stranded DNA. This RNA transcript, which is antisense to the original target mRNA, can act as a template for a further round of NASBA reactions, with primer 2 annealing to the RNA and priming synthesis of the first cDNA strand and primer 1 priming synthesis of the second cDNA strand. The general principles of the NASBA reaction are well known in the art (see Compton, J. Nature. 350: 91-92).

The target-specific probe oligonucleotides described herein may also be attached to a solid support, such as magnetic microbeads, and used as "capture probes" to immobilise the product of the NASBA amplification reaction (a single stranded RNA). The target-specific "molecular beacons" probes described herein may be used for real-time monitoring of the NASBA reaction.

Kits according to the invention may also

including a positive control containing E6 and/or L1 mRNA from a known HPV type. Suitable controls include, for example, nucleic acid extracts prepared from cell lines infected with known HPV types (e.g. HeLa, CaSki).

Kits further may contain internal control amplification primers, e.g. primers specific for human U1A RNA.

Kits containing primers (and optionally probes) suitable for use in NASBA amplification may further comprise a mixture of enzymes required for the NASBA reaction, e.g. enzyme mixture containing an RNA directed DNA polymerase (e.g. a reverse transcriptase), a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA (e.g. RNaseH) and an RNA polymerase. The RNA polymerase should be one which recognises the promoter sequence present in the 5' terminal region of the NASBA P1 primers supplied in the reagent kit. The kit may also comprise a supply of NASBA buffer containing the ribonucleosides and deoxyribonucleosides required for RNA and DNA synthesis. The composition of a standard NASBA reaction buffer will be well known to those skilled in the art (see also Leone et al., *ibid*).

Primers suitable for use in detection of HPV L1 and E6 mRNA by NASBA or PCR are listed in the following tables. However, these are merely illustrative and it is not intended that the scope of the invention should be limited to these specific molecules.

In the following Tables the NASBA P2 primers (p2) include the sequence GATGCAAGGTCGCATATGAG at the 5' end;

the NASBA P1 primers (p1) include the sequence AATTCTAATACGACTCACTATAGGGAGAAGG at the 5' end. Oligonucleotides suitable for use as probes are identified by "po". The P2 primers generally contain HPV sequences from the postive strand, whereas the p1 primers generally contain HPV sequences from the negative strand. nt-refers to nucleotide position in the relevant HPV genomic sequence.

Table 1-E6 NASBA primers and probes

Primer name	Sequence	HPV Type	nt
HAe6701p2	GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAG AAAGTTA	16	116
HAe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGGTT TGTTGTATTGCTGTTC	16	368
HAe6702p2	GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAG AAA	16	116
HAe6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGTTTG TTGTATTGCTGTTC	16	368
HAe6702Ap1	AATTCTAATACGACTCACTATAGGGAGAAGGTCA CGTCGCAGTAACTGT	16	208
HAe6702Bp1	AATTCTAATACGACTCACTATAGGGAGAAGGTTG CTTGCAGTACACACA	16	191
HAe6702Cp1	AATTCTAATACGACTCACTATAGGGAGAAGGTGC AGTACACACATTCTA	16	186
HAe6702Dp1	AATTCTAATACGACTCACTATAGGGAGAAGGGCA GTACACACATTCTAA	16	185
H16e6702Ap2	GATGCAAGGTCGCATATGAGACAGTTATGCACAGAGCT	16	142
H16e6702Bp2	GATGCAAGGTCGCATATGAGATATTAGAATGTGTGTAC	16	182
H16e6702Cp2	GATGCAAGGTCGCATATGAGTTAGAATGTGTGTACTGC	16	185
H16e6702Dp2	GATGCAAGGTCGCATATGAGGAATGTGTGTACTGCAAG	16	188
H16e6702Apo	ACAGTTATGCACAGAGCT	16	142
H16e6702Bpo	ATATTAGAATGTGTGTAC	16	182
H16e6702Cpo	TTAGAATGTGTGTACTGC	16	185
H16e6702Dpo	GAATGTGTGTACTGCAAG	16	188
HAe6701po	CTTTGCTTTTCGGGATTTATGC	16	235
HAe6702po	TATGACTTTGCTTTTCGGGA	16	230
HAe6702mb1	X_2-cgcatgTATGACTTTGCTTTTCGGGAcatgcg -X_3	16	230
HAe6702mb2	X_2-ccagctTATGACTTTGCTTTTCGGGAagctgg -X_3	16	230
HAe6702mb3	X_2-cacgcTATGACTTTGCTTTTCGGGAgcgtg -X_3	16	230

Primer name	Sequence	HPV Type	nt
H16e6702mb4	X_2-cgatcgTATGACTTTGCTTTTCGGGAcgatcg-X_3	16	230
HAe6703p2	GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATGAA ATAGTA	16	656
HAe6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCACAA CCGAAGCGTAGAGTCACAC	16	741
HAe6703po	TGGACAAGCAGAACCGGACAGAGC	16	687
HAe6704p2	GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATGAA ATAGA	16	656
HAe6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCACAA CCGAAGCGTAGAGTCA	16	741
HAe6704po	AGCAGAACCGGACAGAGCCCATTA	16	693
H18e6701p2	GATGCAAGGTCGCATATGAGACGATGAAATAGATGGA GTT	18	702
H18e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACGGA CACACAAAGGACAG	18	869
H18e6701po	AGCCGAACCACAACGTCACA	18	748
H18e6702p2	GATGCAAGGTCGCATATGAGGAAAACGATGAAATAGA TGGAG	18	698
H18e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGACACCA CGGACACACAAAGGACAG	18	869
H18e6702po	GAACCACAACGTCACACAATG	18	752
H18e6702mb1	X_2-cgcatgGAACCACAACGTCACACAATGcatgcg-X_3	18	752
H18e6702mb2	X_2-ccgtcgGAACCACAACGTCACACAATGcgacgg-X_3	18	752
H18e6702mb3	X_2-cggaccGAACCACAACGTCACACAATGggtccg-X_3	18	752
H18e6702mb4	X_2-cgatcgGAACCACAACGTCACACAATGcgatcg-X_3	18	752
H18e6703p2	GATGCAAGGTCGCATATGAGTTCCGGTTGACCTTCTA TGT	18	651
H18e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGTCGT CTGCTGAGCTTTCT	18	817
H18e6704p2	GATGCAAGGTCGCATATGAGGCAAGACATAGAAATAA CCTG	18	179
H18e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGACCCAG TGTTAGTTAGTT	18	379
H18e6704po	TGCAAGACAGTATTGGAACT	18	207
H31e6701p2	GATGCAAGGTCGCATATGAGGGAAATACCCTACGATG AAC	31	164
H31e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGACAC AACGGTCTTTGACA	31	423
H31e6701po	ATAGGGACGACACACCACACGGAG	31	268
H31e6702p2	GATGCAAGGTCGCATATGAGGGAAATACCCTACGATG AACTA	31	164
H31e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCTGGAC ACAACGGTCTTTGACA	31	423
H31e6702po	TAGGGACGACACACCACACGGA	31	269
H31e6703p2	GATGCAAGGTCGCATATGAGACTGACCTCCACTGTTA TGA	31	617
H31e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGTATCTA	31	766

Primer name	Sequence	HPV Type	nt
	CTTGTGTGCTCTGT		
H31e6703po	GACAAGCAGAACCGGACACATC	31	687
H31e6704p2	GATGCAAGGTCGCATATGAGTGACCTCCACTGTTATG AGCAATT	31	619
H31e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGTGCGAA TATCTACTTGTGTGCTCT GT	31	766
H31e6704po	GGACAAGCAGAACCGGACACATCCAA	31	686
H31e6704mb1	X_2-ccgaaggGGACAAGCAGAACCGGACACATCC AAccttcgg -X_3	31	686
H31e6704mb2	X_2-ccgtcgGGACAAGCAGAACCGGACACATCCA Acgacgg -X_3	31	686
H31e6704mb3	X_2-cacgtcgGGACAAGCAGAACCGGACACATCCAA cgacgtg -X_3	31	686
H31e6704mb4	X_2-cgcagcGGACAAGCAGAACCGGACACATCCAA gctgcg -X_3	31	686
H31e6704mb5	X_2-cgatcgGGACAAGCAGAACCGGACACATCCAA cgatcg -X_3	31	686
H31e6705p2	GATGCAAGGTCGCATATGAGACTGACCTCCACTGTTAT	31	617
H31e6705p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACGAT TCCAAATGAGCCCAT	31	809
H33e6701p2	GATGCAAGGTCGCATATGAGTATCCTGAACCAACTGA CCTAT	33	618
H33e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTGACA CATAAACGAACTG	33	763
H33e6701po	CAGATGGACAAGCACAACC	33	694
H33e6703p2	GATGCAAGGTCGCATATGAGTCCTGAACCAACTGACC TAT	33	620
H33e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCCATA AGTAGTTGCTGTAT	33	807
H33e6703po	GGACAAGCACAACCAGCCACAGC	33	699
H33e6703mb1	X_2-ccaagcGGACAAGCACAACCAGCCACAGCgct tgg -X_3	33	699
H33e6703mb2	X_2-ccaagcgGGACAAGCACAACCAGCCACAGC cgcttgg -X_3	33	699
H33e6703mb3	X_2-cccagcGGACAAGCACAACCAGCCACAGCgct ggg -X_3	33	699
H33e6703mb4	X_2-ccaaagcGGACAAGCACAACCAGCCACAGCg ctttgg -X_3	33	699
H33e6703mb5	X_2-cctgcGGACAAGCACAACCAGCCACAGCgcagg -X_3	33	699
H33e6703mb6	X_2-cgatcgGGACAAGCACAACCAGCCACAGCcga tcg -X_3	33	699
H33e6702p2	GATGCAAGGTCGCATATGAGGACCTTTGTGTCCTCAA GAA	33	431
H33e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGGTCA GTTGGTTCAGGATA	33	618

Primer name	Sequence	HPV Type	nt
H33e6702po	AGAAACTGCACTGTGACGTGT	33	543
H35e6701p2	GATGCAAGGTCGCATATGAGATTACAGCGGAGTGAGG TAT	35	217
H35e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCTTT GCTTTTCAACTGGA	35	442
H35e5601po	ATAGAGAAGGCCAGCCATAT	35	270
H35e6702p2	GATGCAAGGTCGCATATGAGTCAGAGGAGGAGGAAGA TACTA	35	655
H35e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGATTAT ~~GGTGTGTGTGAAGA~~	35	844
H35e6703p2	GATGCAAGGTCGCATATGAGCCCGAGGCAACTGACCT ATA	35	610
H35e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCAAT GTGTGTGCTCTGTA	35	770
H35e6702po	GACAAGCAAAACCAGACACCTCCAA	35	692
H35e6703po	GACAAGCAAAACCAGACACC	35	692
H52e6701p2	GATGCAAGGTCGCATATGAGTTGTGTGAGGTGCTGGA AGAAT	52	144
H52e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCCTCT CTTCTAATGTTT	52	358
H52e6701po	GTGCCTACGCTTTTTATCTA	52	296
H52e6702p2	GATGCAAGGTCGCATATGAGGTGCCTACGCTTTTTAT CTA	52	296
H52e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGGGTC TCCAACACTCTGAACA	52	507
H52e6702po	TGCAAACAAGCGATTTCA	52	461
H58e6701p2	GATGCAAGGTCGCATATGAGTCAGGCGTTGGAGACATC	58	157
H58e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGCAAT CGTAAGCACACT	58	301
H58e6702p2	GATGCAAGGTCGCATATGAGTCTGTGCATGAAATCGAA	58	173
H58e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGCACA CTTTACATACTG	58	291
H58e6701po	TGAAATGCGTTGAATGCA	58	192
H58e6702po	TTGCAGCGATCTGAGGTATATG	58	218
HBe6701p2	GATGCAAGGTCGCATATGAGTACACTGCTGGACAACAT	B(11)	514
HBe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCATCT TCTGAGCTGTCT	B(11)	619
HBe6702p2	GATGCAAGGTCGCATATGAGTACACTGCTGGACAACA TGCA	B(11)	514
HBe6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCACA TCCACAGCAACAGGTCA	B(11)	693
HBe6701po	GTAGGGTTACATTGCTATGA	B(11)	590
HBe6702po	GTAGGGTTACATTGCTATGAGC	B(11)	590
HBe6703p2	GATGCAAGGTCGCATATGAGTGACCTGTTGCTGTGGA TGTGA	B(11)	693
HBe6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGTACCTG AATCGTCCGCCAT	B(11)	832

Primer name	Sequence	HPV Type	nt
HBe6703po	ATWGTGTGTCCCATCTGC	B(11)	794
HCe6701p2	GATGCAAGGTCGCATATGAGCATGCCATAAATGTATAGA	C(18 39 45)	295
HCe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACCGC AGGCACCTTATTAA	C(18 39 45	408
HCe6701po	AGAATTAGAGAATTAAGA	C(18 39 45	324
H39e6701p2	GATGCAAGGTCGCATATGAGGCAGACGACCACTACAG CAAA	39	210
H39e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGACACCG AGTCCGAGTAATA	39	344
H39e6701po	ATAGGGACGGGGAACCACT	39	273
H39e6702p2	GATGCAAGGTCGCATATGAGTATTACTCGGACTCGGTGT	39	344
H39e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCTTGGG TTTCTCTTCGTGTTA	39	558
H39e6702po	GGACCACAAAACGGGAGGAC	39	531
H39e6703p2	GATGCAAGGTCGCATATGAGGAAATAGATGAACCCGA CCA	39	703
H39e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCACAC CACGGACACACAAA	39	886
H39e6703po	TAGCCAGACGGGATGAACCACAGC	39	749
H45e6701p2	GATGCAAGGTCGCATATGAGAACCATTGAACCCAGCA GAAA	45	430
H45e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCTTTC TTGCCGTGCCTGGTCA	45	527
H45e6702p2	GATGCAAGGTCGCATATGAGGAAACCATTGAACCCAG CAGAAAA	45	428
H45e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTGCTA TACTTGTGTTTCCCTACG	45	558
H45e6701po	GTACCGAGGGCAGTGTAATA	45	500
H45e6702po	GGACAAACGAAGATTTCACA	45	467
H45e6703p2	GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGTTA CCAGCAAT	45	656
H45e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACCAC GGACACACAAAGGACAAG	45	868
H45e6704p2	GATGCAAGGTCGCATATGAGCTGTTGACCTGTTGTGT TACGA	45	654
H45e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCACGG ACACACAAAGGACAAG	45	868
H45e6705p2	GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGTTA CGA	45	656
H45e6705p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGGAC ACACAAAGGACAAG	45	868
H45e6703po	GAGTCAGAGGAGGAAAACGATG	45	686
H45e6704po	AGGAAAACGATGAAGCAGATGGAGT	45	696
H45e6705po	ACAACTACCAGCCCGACGAGCCGAA	45	730
H51e6701p2	GATGCAAGGTCGCATATGAGGGAGGAGGATGAAGTAG	51	658

Primer name	Sequence	HPV Type	nt
	ATA		
H51e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCCCATTAACATCTGCTGTA	51	807
H51e6702p2	GATGCAAGGTCGCATATGAGAGAGGAGGAGGATGAAGTAGATA	51	655
H51e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGGGCAAACCAGGCTTAGT	51	829
H51e6701po	GCAGGTGTTCAAGTGTAGTA	51	747
H51e6702po	TGGCAGTGGAAAGCAGTGGAGACA	51	771
H56e6701p2	GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGACAAACATCT	56	519
H56e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTCATCCTCATCCTCATCCTCTGA	56	665
H56e6702p2	GATGCAAGGTCGCATATGAGTGGGGTGCTGGAGACAAACATC	56	520
H56e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCATCCTCATCCTCATCCTCTGA	56	665
H56e6703p2	GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGACAAACAT	56	519
H56e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCACAAACTTACACTCACAACA	56	764
H56e6701po	AAAGTACCAACGCTGCAAGACGT	56	581
H56e6702po	AGAACTAACACCTCAAACAGAAAT	56	610
H56e6703po	AGTACCAACGCTGCAAGACGTT	56	583
H56e6703po1	TTGGACAGCTCAGAGGATGAGG	56	656
H56e6704p2	GATGCAAGGTCGCATATGAGGATTTTCCTTATGCAGTGTG	56	279
H56e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGGACATCTGTAGCACCTTATT	56	410
H56e6704po	GACTATTCAGTGTATGGAGC	56	348
HPVAPO1A	CAACTGAYCTMYACTGTTATGA	A (16 31 35)	
HPVApo1Amb1	X_2-cgcatgCAACTGAYCTMYACTGTTATGAcatgcg-X_3	A (16 31 35)	
HPVApo1Amb2	X_2-ccgtcgCAACTGAYCTMYACTGTTATGAcgacgg -X_3	A (16 31 35)	
HPVApo1Amb3	X_2-ccacccCAACTGAYCTMYACTGTTATGAgggtgg -X_3	A (16 31 35)	
HPVApo1Amb4	X_2-cgatcgCAACTGAYCTMYACTGTTATGAcgatcg -X_3	A (16 31 35)	
HPVAPO4A	GAAMCAACTGACCTAYWCTGCTAT	A (33 52 58)	
HPVAPO4Amb1	X_2-ccaagcGAAMCAACTGACCTAYWCTGCTATgcttgg -X_3	A (33 52 58)	
HPVAPO4Amb2	X_2-ccaagccGAAMCAACTGACCTAYWCTGCTAT	A (33	

Primer name	Sequence	HPV Type	nt
	ggcttgg -X_3	52 58)	
HPVAPO4Amb3	X_2-ccaagcgGAAMCAACTGACCTAYWCTGCTA Tcgcttgg -X_3	A (33 52 58)	
HPVAPO4Amb4	X_2-ccagcgGAAMCAACTGACCTAYWCTGCTATcg ctgg -X_3	A (33 52 58)	
HPVAPO4Amb5	X_2-cgatcgGAAMCAACTGACCTAYWCTGCTATcg atcg -X_3	A (33 52 58)	
HPVCPO4	AAGACATTATTCAGACTC	C (18 45 39)	
HPVCPO4Amb1	X_2-ccaagcAAGACATTATTCAGACTCgcttgg -X_3	C (18 45 39)	
HPVCPO4Amb2	X_2-cgcatgAAGACATTATTCAGACTCcatgcg -X_3	C (18 45 39)	
HPVCPO4Amb3	X_2-cccagcAAGACATTATTCAGACTCgctggg -X_3	C (18 45 39)	
HPVCPO4Amb4	X_2-cgatcgAAGACATTATTCAGACTCcgatcg -X_3	C (18 45 39)	

Pairs of P1 and P2 primers having the same prefix (e.g. HAe6701p1 and HAe6701p2) are intended to be used in combination. However, other combinations may also be used, as summarised below for HPV types 16, 18, 31 and 33.

Suitable primer-pairs for amplification of HPV 16 E6 mRNA are as follows:

HAe6701p2 or HAe6702p2 (both nt 116) with HAe6701p1 or HAe6702p1 (both nt 368).

H16e6702Ap2 (nt 142), H16e6702Bp2 (nt 182), H16e6702Cp2 (nt 185) or H16e6702Dp2 (nt 188) with HAe6701p1 or HAe6702p1 (both nt 368).

HAe6701p2 or HAe6702p2 (both nt 116) with HAe6702Ap1 (nt 208), HAe6702Bp1 (nt 191), HAe6702Cp1 (nt 186) or

HAe6702Dp1 (185). These combinations are suitable for amplification of all E6 splice variants.

HAe6703p2 or HAe6704p2 (both nt 656) with HAe6703p1 or HAe6704p1 (both nt 741). These combinations are suitable for amplification of all transcripts containing the E7 coding region (at least up to nt 741).

The following primer-pairs are preferred for amplification of HPV 18 E6 mRNA:

H18e6701p2 (nt 702) or H18e6702p2 (nt 698) with H18e6701p1 or H18e6702p1 (both nt 869).

H18e6703p2 (nt 651) with H18e6703p1 (nt 817).

H18e6704p2 (nt 179) with H18e6704p1 (nt 379).

The following primer-pairs are preferred for amplification of HPV 31 E6 mRNA:

H31e6701p2 or H31e6702p2 (both nt 164) with H31e6701p1 or H31e6702p1 (both nt 423).

H31e6703p2 (nt 617), H31e6704p2 (nt 619) or H31e6705p2 (nt 617) with H31e6703p1 (nt 766), H31e6704p1 (766) or H31e6705p1 (nt 809).

The following primer-pairs are preferred for amplification of HPV 33 E6 mRNA:

H33e6701p2 (nt 618) or H33e6703p2 (nt 620) with H33e6701p1 (nt 763) or H33e6703p1 (nt 807).

H33e6702p2 (nt 431) with H33e6702p1 (nt 618).

Table 2-E6 PCR primers

Primer name	Sequence	HPV type	nt
HAe6701PCR2	CCACAGGAGCGACCCAGAAAGTTA	16	116
HAe6701PCR1	ACGGTTTGTTGTATTGCTGTTC	16	368
HAe6702PCR2	CCACAGGAGCGACCCAGAAA	16	116
HAe6702PCR1	GGTTTGTTGTATTGCTGTTC	16	368
HAe6703PCR2	CAGAGGAGGAGGATGAAATAGTA	16	656
HAe6703PCR1	GCACAACCGAAGCGTAGAGTCACAC	16	741
HAe6704PCR2	CAGAGGAGGAGGATGAAATAGA	16	656
HAe6704PCR1	GCACAACCGAAGCGTAGAGTCA	16	741
H18e6701PCR2	ACGATGAAATAGATGGAGTT	18	702
H18e6701PCR1	CACGGACACACAAAGGACAG	18	869
H18e6702PCR2	GAAAACGATGAAATAGATGGAG	18	698
H18e6702PCR1	ACACCACGGACACACAAAGGACAG	18	869
H18e6703PCR2	TTCCGGTTGACCTTCTATGT	18	651
H18e6703PCR1	GGTCGTCTGCTGAGCTTTCT	18	817
H18e6704PCR2	GCAAGACATAGAAATAACCTG	18	179
H18e6704PCR1	ACCCAGTGTTAGTTAGTT	18	379
H31e6701PCR2	GGAAATACCCTACGATGAAC	31	164
H31e6701PCR1	GGACACAACGGTCTTTGACA	31	423
H31e6702PCR2	GGAAATACCCTACGATGAACTA	31	164
H31e6702PCR1	CTGGACACAACGGTCTTTGACA	31	423
H31e6703PCR2	ACTGACCTCCACTGTTATGA	31	617
H31e6703PCR1	TATCTACTTGTGTGCTCTGT	31	766
H31e6704PCR2	TGACCTCCACTGTTATGAGCAATT	31	619
H31e6704PCR1	TGCGAATATCTACTTGTGTGCTCT GT	31	766
H31e6705PCR2	ACTGACCTCCACTGTTAT	31	617
H31e6705PCR1	CACGATTCCAAATGAGCCCAT	31	809
H33e6701PCR2	TATCCTGAACCAACTGACCTAT	33	618
H33e6701PCR1	TTGACACATAAACGAACTG	33	763
H33e6703PCR2	TCCTGAACCAACTGACCTAT	33	620
H33e6703PCR1	CCCATAAGTAGTTGCTGTAT	33	807
H33e6702PCR2	GACCTTTGTGTCCTCAAGAA	33	431
H33e6702PCR1	AGGTCAGTTGGTTCAGGATA	33	618
H35e6701PCR2	ATTACAGCGGAGTGAGGTAT	35	217
H35e6701PCR1	GTCTTTGCTTTTCAACTGGA	35	442
H35e6702PCR2	TCAGAGGAGGAGGAAGATACTA	35	655
H35e6702PCR1	GATTATGCTCTCTGTGAACA	35	844
H35e6703PCR2	CCCGAGGCAACTGACCTATA	35	610
H35e6703PCR1	GTCAATGTGTGTGCTCTGTA	35	770
H52e6701PCR2	TTGTGTGAGGTGCTGGAAGAAT	52	144

Primer name	Sequence	HPV type	nt
H52e6701PCR1	CCCTCTCTTCTAATGTTT	52	358
H52e6702PCR2	GTGCCTACGCTTTTTATCTA	52	296
H52e6702PCR1	GGGGTCTCCAACACTCTGAACA	52	507
H58e6701PCR2	TCAGGCGTTGGAGACATC	58	157
H58e6701PCR1	AGCAATCGTAAGCACACT	58	301
H58e6702PCR2	TCTGTGCATGAAATCGAA	58	173
H58e6702PCR1	AGCACACTTTACATACTG	58	291
HBe6701PCR2	TACACTGCTGGACAACAT	B(11)	514
HBe6701PCR1	TCATCTTCTGAGCTGTCT	B(11)	619
HBe6702PCR2	TACACTGCTGGACAACATGCA	B(11)	514
~~HBe6702PCR1~~	~~GTCACATCGACAGGAACAGGTCA~~	~~B(11)~~	~~693~~
HBe6703PCR2	TGACCTGTTGCTGTGGATGTGA	B(11)	693
HBe6703PCR1	TACCTGAATCGTCCGCCAT	B(11)	832
HCe6701PCR2	CATGCCATAAATGTATAGA	C (18 39 45	295
HCe6701PCR1	CACCGCAGGCACCTTATTAA	C (18 39 45	408
H39e6701PCR2	GCAGACGACCACTACAGCAAA	39	210
H39e6701PCR1	ACACCGAGTCCGAGTAATA	39	344
H39e6702PCR2	TATTACTCGGACTCGGTGT	39	344
H39e6702PCR1	CTTGGGTTTCTCTTCGTGTTA	39	558
H39e6703PCR2	GAAATAGATGAACCCGACCA	39	703
H39e6703PCR1	GCACACCACGGACACACAAA	39	886
H45e6701PCR2	AACCATTGAACCCAGCAGAAA	45	430
H45e6701PCR1	TCTTTCTTGCCGTGCCTGGTCA	45	527
H45e6702PCR2	GAAACCATTGAACCCAGCAGAAAA	45	428
H45e6702PCR1	TTGCTATACTTGTGTTTCCCTACG	45	558
H45e6703PCR2	GTTGACCTGTTGTGTTACCAGCAAT	45	656
H45e6703PCR1	CACCACGGACACACAAAGGACAAG	45	868
H45e6704PCR2	CTGTTGACCTGTTGTGTTACGA	45	654
H45e6704PCR1	CCACGGACACACAAAGGACAAG	45	868
H45e6705PCR2	GTTGACCTGTTGTGTTACGA	45	656
H45e6705PCR1	ACGGACACACAAAGGACAAG	45	868
H51e6701PCR2	GGAGGAGGATGAAGTAGATA	51	658
H51e6701PCR1	GCCCATTAACATCTGCTGTA	51	807
H51e6702PCR2	AGAGGAGGAGGATGAAGTAGATA	51	655
H51e6702PCR1	ACGGGCAAACCAGGCTTAGT	51	829
H56e6701PCR2	TTGGGGTGCTGGAGACAAACATCT	56	519
H56e6701PCR1	TTCATCCTCATCCTCATCCTCTGA	56	665
H56e6702PCR2	TGGGGTGCTGGAGACAAACATC	56	520
H56e6702PCR1	CATCCTCATCCTCATCCTCTGA	56	665
H56e6703PCR2	TTGGGGTGCTGGAGACAAACAT	56	519
H56e6703PCR1	CCACAAACTTACACTCACAACA	56	764
H56e6704PCR2	GATTTTCCTTATGCAGTGTG	56	279
H56e6704PCR1	GACATCTGTAGCACCTTATT	56	410

Preferred PCR primer-pairs for HPV types 16, 18, 31 and 33 are analogous to the NASBA primer-pairs.

Table 3-L1 NASBA primers and probes

Primer name	**Sequence**
Onc2A2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGGGTAA 3'
Onc2A1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCCCCATGTC 3'
Onc2PoA	5' TTGTTACTGTTGTTGATACTAC 3'
Onc2B2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGSRHAA 3'
Onc2B1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCMMCATGDC 3'
Onc2PoB	5' TTGTTACTGTTGTTGATACYAC 3'
Onc2PoC	5' TTGTTACTGTTGTTGATACCAC 3'
Onc2C2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGSIIAA 3'
Onc2D2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGIIHAA 3'
Onc2E2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGIRIAA 3'
Onc2F2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGGGTAA 3'
Onc2G2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGGGAAA 3'
Onc2H2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGCATAA 3'
Onc2I2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGGGCAA 3'
Onc2J2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGCACAA 3'
Onc2K1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCMICATGIC 3'
Onc2L1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCAACATGIC 3'
Onc2M1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGTC 3'
Onc2N1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGGC 3'
Onc2O1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGAC 3'
Onc2P1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGCC 3'

Table 4-L1 PCR primers

Primer name	**Sequence**
Onc2A1-PCR	5' AATGGCATTTGTTGGGGTAA 3'
Onc2A2-PCR	5' TCATATTCCTCCCCATGTC 3'

Onc2B1-PCR	5' AATGGCATTTGTTGGSRHAA 3'
Onc2B2-PCR	5' TCATATTCCTCMMCATGDC 3'
Onc2C1-PCR	5' AATGGCATTTGTTGGSIIAA 3'
Onc2D1-PCR	5' AATGGCATTTGTTGGIIHAA 3'
Onc2E1-PCR	5' AATGGCATTTGTTGGIRIAA 3'
Onc2F1-PCR	5' AATGGCATTTGTTGGGGTAA 3'
Onc2G1-PCR	5' AATGGCATTTGTTGGGGAAA 3'
Onc2H1-PCR	5' AATGGCATTTGTTGGCATAA 3'
Onc2I1-PCR	5' AATGGCATTTGTTGGGGCAA 3'
Onc2J1-PCR	5' AATGGCATTTGTTGGCACAA 3'
Onc2K2-PCR	5' TCATATTCCTCMICATGIC 3'
Onc2L2-PCR	5' TCATATTCCTCAACATGIC 3'
Onc2M2-PCR	5' TCATATTCCTCIICATGTC 3'
Onc2N2-PCR	5' TCATATTCCTCIICATGGC 3'
Onc2O2-PCR	5' TCATATTCCTCIICATGAC 3'
Onc2P2-PCR	5' TCATATTCCTCIICATGCC 3'

The HPV-specific sequences in primers Onc2A2/Onc2A1-PCR and Onc2A1/Onc2A2-PCR are identical to fragments of the HPV type 16 genomic sequence from position 6596-6615 (Onc2A2/Onc2A1-PCR), and from position 6729 to 6747(Onc2A1/Onc2A2-PCR).

The HPV-specific sequences in Onc2B2/Onc2B1-PCR and Onc2B1/Onc2B2-PCR are variants of the above sequences, respectively, including several degenerate bases. Representations of the sequences of degenerate oligonucleotide molecules provided herein use the standard IUB code for mixed base sites: N=G,A,T,C; V=G,A,C; B=G,T,C; H=A,T,C; D=G,A,T; K=G,T; S=G,C;W=A,T; M=A,C; Y=C,T; R=A,G.

It is also possible to use variants of the HPV-specific sequences in Onc2B2/Onc2B1-PCR and Onc2B1/Onc2B2-PCR wherein any two of nucleotides "SRH"

towards the 3' end of the sequence are replaced with inosine (I), as follows:

5' AATGGCATTTGTTGGIIHAA 3'

5' AATGGCATTTGTTGGSIIAA 3'

5' AATGGCATTTGTTGGIRIAA 3'

The HPV-specific sequences in Onc2C2, Onc2D2, Onc2E2, Onc2F2, Onc2G2, Onc2H2, Onc2I2, Onc2J2, Onc2C1-PCR, Onc2D1-PCR, Onc2E1-PCR, Onc2F1-PCR, Onc2G1-PCR, Onc2H1-PCR, Onc2I1-PCR and Onc2J1-PCR are variants based on the HPV-specific sequence in Onc2B2/Onc2B1-PCR, whereas the HPV-specific sequences in Onc2K1, Onc2L1, Onc2M1, Onc2N1, Onc2O1, Onc2P1, Onc2K2-PCR, Onc2L2-PCR, Onc2M2-PCR, Onc2N2-PCR, Onc2O2-PCR and Onc2P2-PCR are variants based on the HPV-specific sequence in Onc2B1/Onc2B2-PCR. These variants include degenerate bases and also inosine (I) residues. This sequence variation enables oligonucleotides incorporating the variant sequences to bind to multiple HPV types. Inosine bases do not interfere with hybridization and so may be included at sites of variation between HPV types in order to construct a "consensus" primer able to bind to multiple HPV types.

Any one or more of primers Onc2A2, Onc2B2, Onc2C2, Onc2D2, Onc2E2, Onc2F2, Onc2G2, Onc2H2, Onc2I2 and Onc2J2, may be used in combination with any one or more of primers Onc2A1, Onc2B1, Onc2K1, Onc2L1, Onc2M1, Onc2N1, Onc2O1 and Onc2P1, for NASBA amplification of HPV L1 mRNA.

Any one or more of primers Onc2A1-PCR, Onc2B1-PCR, Onc2C1-PCR, Onc2D1-PCR, Onc2E1-PCR, Onc2F1-PCR,

Onc2G1-PCR, Onc2H1-PCR, Onc2I1-PCR and Onc2J1-PCR, may be used in combination with any one or more of primers Onc2A2-PCR, Onc2B2-PCR, Onc2K2-PCR, Onc2L2-PCR, Onc2M2-PCR, Onc2N2-PCR, Onc2O2-PCR and Onc2P2-PCR for PCR amplification of HPV L1 mRNA.

The invention will be further understood with reference to the following experimental examples:

Example 1-Detection of HPV mRNA by NASBA-based nucleic acid amplification and real-time detection

Collection and preparation of clinical samples

Pap smears and HPV samples were collected from 5970 women in the cervical screening program in Oslo. Samples intended for RNA/DNA extraction were treated as follows:

A cervical cytobrush sample was collected in 9 ml lysis buffer (5M Guanidine thiocyanate) from each women attending the cervical screening program. Since RNA is best protected in the 5M guanidine thiocyanate at -70°C only 1 ml of the total volume of sample was used for each extraction round. 2-3 tubes with the RNA/DNA were stored at -167°C and the rest was stored at -70°C.

RNA and DNA were automatically isolated from 5300 women in the first round of extraction. 2 or more ml was extracted from the samples that were HPV PCR positive. The RNA and DNA were extracted according to the "Booms" isolation method from Organon Teknika (Organon Teknika B.V., Boselind 15, P.O. Box 84, 5280 AB Baxtel, The Netherlands; now Biomérieux, 69280 Marcy l'Etoile, France).

Amplification

Precautions for avoiding contamination:

1. Perform nucleic acid release, isolation and amplification/detection in separate laboratory areas.

2. Store and prepare reagents for nucleic acid release, isolation and amplification/detection at the laboratory areas where nucleic acid release, isolation and amplification/detection are to be performed, respectively.

3. Keep all tubes and vials closed when not in use.

4. Pipettes and other equipment that have been used in one laboratory area must not be used in the other areas.

5. Use a fresh pipette or pipette tip for each pipetting action.

6. Use pipettes with aerosol resistant tips for fluids possibly containing nucleic acid. Pipetting of solutions must always be performed out of or into an isolated tube that is opened and closed exclusively for this action. All other tubes and vials should be kept closed and separated from the one handled.

7. Use disposable gloves when working with clinical material possibly containing target-RNA or amplified material. If possible, change gloves after each pipetting step in the test procedure, especially after contact with possibly contaminated material.

8. Collect used disposable material in a container. Close and remove container after each test run.

9. Soak tube racks used during nucleic acid isolation or amplification/detection in a detergent (e.g. Merck Extran MA01 alkaline) for at least one hour after each test run.

The following procedure was carried out using reagents from the Nuclisens™ Basic Kit, supplied by Organon Teknika.

Procedure for n=10 samples:-

1. Prepare enzyme solution.

Add 55 µl of enzyme diluent (from Nuclisens™ Basic Kit; contains sorbitol in aqueous solution) to each of 3 lyophilized enzyme spheres (from Nuclisens™ Basic Kit; contains AMV-RT, RNase H, T7 RNA polymerase and BSA). Leave this enzyme solution at least for 20 minutes at room temperature. Gather the enzyme solutions in one tube, mix well by flicking the tube with your finger, spin down briefly and use within 1 hour. Final concentrations in the enzyme mix are 375 mM sorbitol, 2.5 µg BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-reverse transcriptase.

2. Prepare reagent sphere/KCl solution.

For 10 samples: add 80 µl reagent sphere diluent (from Nuclisens™ Basic Kit; contains Tris/HCl (pH 8.5), 45% DMSO) to the lyophilized reagent sphere (from Nuclisens™ Basic Kit; contains nucleotides, dithiotreitol and $MgCl_2$) and immediately vortex well. Do this with 3 reagent spheres and mix the solutions in one tube.

Add 3 µl NASBA water (from Nuclisens™ Basic Kit) to the reconstituted reagent sphere solution and mix well.

Add 56 µl of KCl stock solution (from Nuclisens™ Basic Kit) and mix well. Use of this KCl/water mixture will result in NASBA reactions with a final KCl

concentration of 70 mM. Final concentrations in the reagent/KCl solution are 1 mM of each dNTP, 2 mM of ATP, UTP and CTP, 1.5 mM GTP, and 0.5 mM ITP, 0.5 mM dithiotreitol, 70 mM KCl, 12 mM $MgCl_2$, 40 mM Tris-HCl (pH 8.5).

3. Prepare primer/probe solution containing target-specific primers and molecular beacon probe.

For each target reaction transfer 91 μl of the reagent sphere/KCl solution (prepared in step 2) into a fresh tube. Add 25 μl of primers/molecular beacon probe solution (to give final concentration of ~0.1-0.5 μM each of the sense and antisense primers and ~ 15-70 pmol molecular beacon probe per reaction). Mix well by vortexing. Do not centrifuge.

In case less than 10 target RNA amplifications are being performed refer to the table below for the appropriate amounts of reagent sphere solution, KCl/water solution and primers to be used. Primer solutions should be used within 30 minutes after preparation.

Reactions (n)	Reagent sphere solution (μl)	KCl/water (μl)	Primer mix (μl)
10	80	30	10
9	72	27	9
8	64	24	8
7	56	21	7
6	48	18	6
5	40	15	5
4	32	12	4
3	24	9	3
2	16	6	2
1	8	3	1

4. Addition of samples

For each target RNA reaction:

In a 96 well microtiter plate pipette 10 μl of the primer/probe solution (prepared in step 3) into each of 10 wells. Add 5 μl nucleic acid extract to each well. Incubate the microtiter plate for 4 minutes at 65 ± 1 °C. Cool to at 41 ± 0.5 °C for 4 minutes. Then to each well add 5 μl enzyme solution. Immediately place the microtiter plate in a fluorescent detection instrument (e.g. NucliSens™ EasyQ Analyzer) and start the amplification.

Results from clinical study

Table 5 shows the distribution of real-time NASBA HPV positive (L1 and/or E6 expression) and PCR HPV positive cases related to cytology results. PCR amplification was carried out as described by Karlsen *et al.*, J Clin Microbiol. 34: 2095-2100, 1996. The figures for expected histology are based on average results from similar study on CIN III lesions (Clavel *et al.*, Br J Cancer, 84: 1616-1623, 2001). The results from several example cases are listed in Table 6.

Table 5

	Normal	**Benign**	**Condyloma**	**CIN III**
Cytology	4474	66	16	15
PCR	9.0%	44.6%	87.5%	73.3%
Real-time NASBA	0.74%	24.6%	37.5%	73.3%
Expected Histology	0.2%	5-15%	15-20%	71%

Table 6

Internal No.	**Cytology**	**PCR**	**L1 NASBA**	**E6 NASBA**
84	Neg	Neg	Neg	31
289	Neg	31	Pos	31
926	Neg	Neg	Pos	16
743	Benign	Neg	Neg	33
1512	Benign	16	Pos	16
3437	Benign	Neg	Neg	18
3696	Benign	16	Pos	Neg
2043	Condyloma	16, 51	Pos	16
3873	Condyloma	16, 51	Pos	16
3634	CIN II	33	Neg	33
4276	CIN III	Neg	Neg	18
4767	CIN III	18	Neg	18
1482	CIN III	Neg	Pos	16
5217	CIN III	31	Neg	31
4696	CIN III	Neg	Neg	Neg

<u>Example 2-Sensitivity of real-time NASBA on control cell lines</u>

Cervical cancer cell lines, CaSki, SiHa and HeLa were diluted in lysis buffer either before automated extraction of nucleic acids using the Boom's extraction method from Organon Teknika/bioMerieux (parallels 1 and 3), or after nucleic acid extraction (parallel 2). Real-time NASBA was performed using molecular beacons probes labelled with Texas red (16, L1 and 18) or FAM (U1A, 33 and 31) following the protocol described above.

Table 7

	CaSki						CaSki						HeLa					
Primer sets and probes	16 E6	U1	16 E6	U1	16 E6	U1	L1	33 E6	L1	33 E6	L1	33 E6	18 E6	31 E6	18 E6	31 E6	18 E6	31 E6
Parallels	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3
Number of Cells																		
100 000	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
10 000	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
1 000	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
100	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
10	+	+	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	-
1	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+	-	+	-
10^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Thus, it is possible to detect HPV E6 mRNA in less than 1 cell using real-time NASBA.

Claims:

1. An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or E6 mRNA are scored as being at risk of developing cervical carcinoma.

2. An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts of the L1 gene of HPV and mRNA transcripts of the E6 gene of HPV, and sorting the subject into one of four categories of risk for development of cervical carcinoma based on expression of L1 and/or E6 mRNA according to the following classification:

Risk category 1: subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68;

Risk category 2: subjects positive for expression of L1 mRNA and positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68;

Risk category 3: subjects positive for expression of L1 mRNA but negative for expression of E6 mRNA;

Risk category 4: subjects negative for expression of L1 mRNA and negative for expression of E6 mRNA.

3. A method according to claim 1 which further comprises screening for expression of p16^{ink4a}, wherein subjects positive for expression of L1 and/or E6 mRNA and positive for expression of p16^{ink4a} are scored as being at risk of developing cervical carcinoma.

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4. An *in vitro* method of screening human subjects for the presence of integrated HPV, which method comprises screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA are scored as carrying integrated HPV.

5. A method according to any one of claims 1 to 4 which comprises screening for L1 mRNA expression using a technique which is able to detect L1 mRNA from substantially all known HPV types.

6. A method according to any one of claims 1 to 5 which comprises screening for E6 mRNA expression using a technique which is able to detect E6 mRNA from at least one cancer-associated HPV type,

7. A method according to claim 6 which comprises screening for E6 mRNA expression using a technique which is able to detect E6 mRNA from HPV types 16, 18, 31 and 33.

8. A method according to any one of claims 1 to 7 wherein screening for L1 and/or E6 mRNA expression is carried using an amplification reaction to amplify

of a region of the mRNA, together with real-time detection of the products of the amplification reaction.

9. A method according to claim 8 wherein screening for L1 and/or E6 mRNA expression is carried using real-time NASBA.

10. A kit for use in the detection of mRNA transcripts of the L1 and E6 genes of HPV, the kit comprising at least one primer-pair suitable for use in amplification of a region of L1 transcripts from at least HPV types 16, 18, 31 and 33 and one or more primer-pairs which enable amplification of a region of E6 transcripts from HPV types 16, 18, 31 and 33.